Epoxy-Keto Derivative of Linoleic Acid Stimulates Aldosterone Secretion

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Abstract—Plasma levels of aldosterone are not always predictable from the activity of renin and the concentration of potassium. Among the unexplained are elevated levels of aldosterone in some obese humans. Obesity is characterized by increased plasma fatty acids and oxidative stress. We postulated that oxidized fatty acids stimulate aldosteroneogenesis. The most readily oxidized fatty acids are the polyunsaturated, and the most abundant of those is linoleic acid. We tested oxidized derivatives of linoleic acid for effects on rat adrenal cells. One derivative, 12,13-epoxy-9-keto-10(trans)-octadecenoic acid (EKODE), was particularly potent. EKODE stimulated aldosteroneogenesis at concentrations from 0.5 to 5 μmol/L, and inhibited aldosteroneogenesis at higher doses. EKODE’s stimulatory effect was most prominent when angiotensin and potassium effects were submaximal. The lipid’s mechanism of action was on the early pathway leading to pregnenolone; its action was inhibited by atrial natriuretic peptide. Plasma EKODE was measured by liquid chromatography/mass spectrometry. All human plasmas tested contained EKODE in concentrations ranging from $10^{-9}$ to $5\times10^{-7}$ mol/L. In samples from 24 adults, levels of EKODE correlated directly with aldosterone ($r=0.53, P=0.007$). In the 12 blacks in that cohort, EKODE also correlated with body mass index and systolic pressure. Those other correlations were not seen in white subjects. The results suggest that oxidized derivatives of polyunsaturated fatty acids other than arachidonic are biologically active. Compounds like EKODE, derived from linoleic acid, may affect adrenal steroid production in humans and mediate some of the deleterious effects of obesity and oxidative stress, especially in blacks. (Hypertension. 2004;43[part 2]:358-363.)

Key Words: fatty acids • oxidative stress • hypertension • aldosterone • obesity • adrenal gland

Aldosterone production by adrenal cells in vitro can be affected by more than 20 hormones, autacoids, ions, and nutrients. It is probable, therefore, that regulation of aldosterone secretion in intact animals and humans is more complex than classical schemes that involve primarily angiotensin II and potassium. In fact, several authors have invoked unknown factors to explain levels of aldosterone that do not comply with predictions based on classical regulators of the adrenal. We encountered one such inexplicable situation when we found that aldosterone levels were higher in some subjects with visceral obesity and hypertension than in lean normotensive subjects, even when we took into account the levels of renin activity and potassium.

Obesity and hypertension are associated with relatively high levels of fatty acids and increased oxidative stress. These facts led us to postulate that oxidized derivatives of fatty acids might drive aldosterone secretion and possibly explain the relatively high levels of aldosterone in some subjects with visceral obesity. The most readily oxidized fatty acids are polyunsaturated, and the most prevalent polyunsaturated fatty acid in humans is linoleic acid. Therefore, we focused our attention on the properties of oxidized derivatives of linoleic acid. We showed that incubating linoleic acid (C18:2, n-6) with rat hepatocytes produced one or more compounds that stimulated aldosterone production by rat adrenal cells. Rat hepatocytes did not produce enough of the oxidized products to characterize, so we used a cell-free method to generate a mixture of milligram quantities of oxidized products of linoleic acid. We fractionated this mixture by high-pressure liquid chromatography (HPLC) and bioassayed the fractions in rat adrenal cells. The most active derivatives were further purified and their structures determined by mass spectroscopy. We were then able to confirm that at least one of the linoleic acid derivatives produced in our cell-free system—the most active one—was produced by rat hepatocytes in vitro. This paper reports some of the adrenal effects and interactions of the most potent and most prevalent of the oxidized products of linoleic acid formed in our system, 12,13-epoxy-9-keto-10(trans)-octadecenoic acid (EKODE), and the results of a single comparison of plasma levels of EKODE and aldosterone in 24 humans. EKODE is a derivative of linoleic acid that has been described before, but its existence and effects in mammals were unexplored.
Methods

EKODE Synthesis

Linoleic acid was oxidized in two steps. First, linoleic acid was incubated with soybean lipoxygenase Type V (Sigma, St. Louis, Mo) to produce 13(S)-hydroperoxide, which was partially purified by silicic acid chromatography eluted with acetone:hexane. The hydroperoxide was then exposed to ferric ion and cysteine, which catalyzed rearrangements with free radical intermediates. The stable products were extracted into chloroform and partially purified by a second silicic acid column. The fractions were tested for their effects on rat adrenals, and the active fractions were purified by reversed-phase HPLC using a C-18 column eluted with a gradient of 65% to 75% methanol in water acidified with 0.1% acetic acid. The column was monitored by ultraviolet absorbance at 234 nm. EKODE eluted at a polarity index of 7.381 (67.7% methanol). It was repurified over the same column using a shallower gradient, dried, and stored as aliquots under argon at −70°C.

The quantity of EKODE in the final extract and aliquots was determined by 3 techniques: gravimetric measurement; absorbance at 234 nm assuming a molar extinction coefficient of 16 500; and isotope dilution using a trace of 13C-labeled linoleic acid of known specific activity in the synthetic protocol.

EKODE in plasma was extracted into hexane:isopropanol:1N citric acid (10:40:1). We determined that citric acid did not cleave the epoxide bond while it protonated the carboxyl group. To control for recovery, we added deuterated EKODE (2H, EKODE), 4 ng per 0.2 mL of plasma. Deuterated EKODE was produced from deuterated linoleic acid (generously supplied by Dr. Richard Adloff, National Center for Agricultural Utilization Research, Peoria, Ill) using the lipoxygenase/iron-cysteine reactions described above. The quantity of EKODE in each extract was determined by liquid chromatography/electrospray ionization mass spectrometry (LC/MS). The molecular weight of EKODE is 310, so the chromatographic eluate was monitored at masses 311 and 315 m/z, representing the m+1 ions of EKODE and deuterated EKODE produced by positive ion mass spectrometry.

Bioassays were performed using rat adrenal glomerulosa cells isolated from capsules by collagenase digestion. EKODE and other lipids were dissolved in ethanol. Incubations were 2 hours at 37°C. Incubation medium was 10% Medium 199 (Sigma #5017) in HEPES-buffered balanced salt solution. The final concentration of potassium was 3.6 mmol/L and of calcium was 0.184 mmol/L unless otherwise noted. Ethanol concentrations never exceeded 1%. No albumin was added to the incubation medium. Aldosterone in the supernatant was measured by radioimmunoassay, using antibody from ICN Biomedicals (Irvine, Calif) and labeled aldosterone from Perkin-Elmer Life Sciences (Boston, Mass). EKODE had no effect when added directly to radioimmunoassays of aldosterone or pregnenolone (data not shown).

The human subjects whose plasma was examined in this experiment were participating in a detailed study of obesity and hypertension, the design and results of which are described in other publications. In brief, 24 adult subjects between the ages 24 and 48 were enrolled; 12 were white, 12 black. Half of each group were men; half were women. Half of the subjects were lean normotensives, and half were obese hypertensives. Although the average blood pressures of the obese subjects were higher than the lean, none of the screening blood pressures exceeded 159/99 mm Hg, and none of the subjects had taken antihypertensive medication for 2 weeks. None of the subjects was a smoker at the time of the study. Adiposity was reported as body mass index. Fat distribution was not assessed at the time when plasma was drawn. The plasma samples we measured were collected on the first day of the study, while subjects were on their usual diets but had fasted overnight and were supine for 30 minutes before venipuncture. Plasma was separated immediately and stored frozen. The clinical experiment and plasma measurements were approved by the institutional review boards at the Medical University of South Carolina and the University of Wisconsin.

Plasma aldosterone was measured by solid-phase radioimmunoassay using antibody-coated tubes from Diagnostic Products Corp. Plasma renin activity was assessed by measuring angiotensin I by radioimmunoassay after incubation under conditions described in the kit purchased from Perkin-Elmer Life Sciences.

Statistical analysis of correlations within the clinical data were assessed by Pearson and Spearman analyses. For the former, EKODE values were entered as the logarithm.

Results

Effects of EKODE on Rat Adrenal Glomerulosa Cells

The structural formula of EKODE is depicted in Figure 1. Figure 2A shows the response of rat adrenal glomerulosa cells to increased concentrations of EKODE in the presence of different ambient concentrations of potassium. These data are redrawn in Figure 2B as a family of dose-response curves.
to potassium when different amounts of EKODE were present in the incubation medium. EKODE exerted its greatest effect when the potassium concentration was low; potassium effect was essentially unaffected by the presence of EKODE until concentrations greater than 5 μmol/L were tested. Effects of higher concentrations of EKODE are described below.

Figure 3A and 3B show the interaction of EKODE and angiotensin II. As in the case of potassium, EKODE’s effect was visible only when angiotensin doses were submaximal. EKODE did not add a stimulatory effect to the effects of high doses of angiotensin. Similarly, EKODE did not add to maximal stimulation by dibutyryl cAMP (dbcAMP) (see below).

EKODE stimulated the “early pathway of aldosteronogenesis,” the pathway that includes signal transduction and the steroidogenic steps between cholesterol and pregnenolone, (Figure 4). When steroidogenesis was blocked at the level of pregnenolone by trilostane, EKODE stimulated formation of that intermediate to the same extent it stimulated the complete pathway to aldosterone when no inhibitor was present. The lack of EKODE effect on the late pathway is illustrated by the data in Figure 5. When aldosteronogenesis was maximized by addition of intermediates closer to aldosterone than pregnenolone, EKODE had no stimulatory effect.

It should be noted that the effect of EKODE is biphasic, stimulating aldosterone production at concentrations between 0.5 and 5 μmol/L, and inhibiting at concentrations above that (Figure 4 and 5). The inhibitory effect is evident when steroidogenesis is maximally stimulated by steroid precursors, dbcAMP, high concentrations of potassium (Figure 5), or angiotensin (data not shown). The inhibitory effect of high concentrations of EKODE is also evident when steroidogenesis is blocked at the level of pregnenolone (Figure 4).

The stimulatory effect of EKODE was neutralized by addition of atrial natriuretic peptide (ANP; Figure 6). Inhibitors of angiotensin receptors did not alter the response of rat adrenal cells to EKODE (data not shown).

Figure 3. Interaction of EKODE and angiotensin II in rat adrenal glomerulosa cells. Cell preparation, incubation and aldosterone assays are described in the text, the legend to Figure 2, and Reference 15. A and B display the same data in different ways. Numbers labeling the curves are the concentrations of angiotensin II (A) or EKODE (B) in the medium. In B, data for the lowest concentration of EKODE (0.15 μmol/L) are omitted.

Figure 4. Comparison of EKODE effects on the early pathway and overall pathway of aldosterone biosynthesis by rat adrenal cells. Cell preparation and incubation are described in the text, the legend to Figure 2, and Reference 15. The early pathway was assessed by measuring pregnenolone in a medium containing trilostane, 10 μmol/L, an inhibitor of 3β-hydroxysteroid dehydrogenase. Aldosterone and pregnenolone were measured by radioimmunoassays with no significant cross-reactivity for the other steroid.

**EKODE Levels and Correlations in Human Subjects**

EKODE was detected in every human plasma sample examined. The mean level was 5×10⁻⁴ mol/L, the range was from 10⁻⁹ to 5×10⁻⁷ mol/L. Concentrations of EKODE correlated directly with levels of aldosterone in plasmas from 24 human

Figure 5. Comparison of EKODE effects on the late pathway and overall pathway of aldosterone biosynthesis by rat adrenal cells and on aldosterone production stimulated by potassium and dibutyryl cAMP (dbcAMP). Cell preparation and incubation are described in the text, the legend to Figure 2, and Reference 15. The late pathway was assessed by measuring aldosterone production in a medium containing trilostane (10 μmol/L) to block the early pathway and steroid precursors beyond pregnenolone. Depicted are the results with progesterone (Progest) and corticosterone (Cortico), each added to a concentration of 3 μmol/L. Similar results were obtained when pregnenolone was added (data not shown). Also shown are the effects of EKODE on cells stimulated with potassium (7.2 mmol/L) and dbcAMP (1 mmol/L). Although EKODE did not stimulate aldosterone production when these stimuli were present, high doses of the lipid were inhibitory to all agonists acting on the early pathway.
subjects. This is shown in Figure 7, where the EKODE values are transformed to their logarithms. The simple correlation coefficient for the group as a whole was $r=0.57$, $P=0.004$ by Pearson analysis of the values and $r=0.53$, $P=0.007$ by Spearman rank analysis.

We observed a racial difference in the correlations between EKODE and other variables. The EKODE:aldosterone relationship was stronger among the 12 blacks than the 12 whites (0.43 versus 0.32 by Spearman rank analysis), although neither group displayed a statistically significant correlation when analyzed separately. In addition, EKODE levels correlated directly with systolic blood pressure in blacks, ($r=0.56$, $P=0.05$ by Spearman rank analysis) but not in whites. The only significant relationship between EKODE levels and adiposity was found among the black subjects in whom the Spearman correlation coefficient between EKODE and body mass index was $r=0.71$, $P=0.006$. There was no statistical relationship between EKODE and BMI in whites.

**Discussion**

Aside from the eicosanoids, oxidized derivatives of polyunsaturated fatty acids have not been extensively studied in humans and other mammals. The reactions producing these derivatives add oxygen to the fatty acid; they do not result in energy-yielding “beta-oxidation.” Polynsaturated fatty acids are quite susceptible to reactions with oxygen, yielding epoxides, ketones, hydroxylated derivatives, and a variety of cleaved products. Some oxidized derivatives of linoleic acid are called leukotoxins because they are formed by leukocytes and are toxic to mammalian tissues. Oxidized derivatives of unsaturated fatty acids can be formed nonenzymatically, as in the spoilage of vegetable oils. Enzyme-catalyzed oxidation usually begins with the action of a lipoxygenase, as the result of which molecular oxygen adds to one side of a double bond to form a hydroperoxide. If the starting material is linoleic acid (C18:2, n-6), and the lipoxygenase is 15-lipoxygenase, the initial product is 13-hydroperoxy-octadecadienoic acid. (This numerical confusion results from the fact that the enzyme was named for the carbon it usually attacks in 20 carbon fatty acids like arachidonic.) The fatty acid hydroperoxide is readily rearranged and further oxidized to more stable products. These steps involve free radicals and can be accelerated by free radical generators such as ferric iron and cysteine.

The principal product of our reaction scheme with linoleic acid contains both epoxide and o xo- (ketone) oxygens in addition to the carboxyl group, with one remaining double bond. Its structure is 12,13-epoxy-9-keto-10(trans)-octadecenoic acid, (EKODE). This unusual compound was not only one of the most prevalent products of our synthesis, it proved to be one of the most active in adrenal cells. We have found EKODE in the plasma of 26 humans, dozens of rats and mice, and 14 Rhesus monkeys. However, we do not know how or where the EKODE in plasma is formed. Some of it may, in fact, originate in foodstuffs.

EKODE did not add to the stimulatory effect of high doses of angiotensin II in vitro. This suggests that any regulatory role it might have in humans would be greatest when renin activity is low. When renin activity is suppressed by high dietary sodium intake, for example, any stimulus of aldosterone secretion becomes particularly relevant to the pathogenesis of cardiovascular disease. We found that the correlation between visceral obesity and plasma aldosterone was strongest when subjects ingested a high-salt diet and had very low plasma renin activities. This observation may have relevance to the relationship between obesity and blood pressure, but it would relate to our current in vitro results only if visceral obesity increased EKODE production and EKODE increased aldosterone production.

EKODE stimulates production of corticosterone by rat adrenal zona fasciculata cells, in addition to its effect on aldosterone production by the zona glomerulosa. In both zones, the lipid’s effect appears to be on the early pathway of steroidogenesis, a series of reactions that includes signal
transduction and produces pregnenolone. This is consistent with inhibition of EKODE by ANP, a peptide inhibitor of signal transduction.\(^2\) A likely mechanism of action for EKODE is to increase intracellular calcium (M. Payet, personal communication, 2003).

The biphasic shape of the EKODE dose-response curve is unusual and puzzling. Inhibition of the adrenal by high concentrations may help explain the inappropriately low aldosterone levels in some dire clinical conditions where levels of nonesterified fatty acids are high.\(^2\)\(^3\)

A stimulation of aldosterone production in rat adrenal cells can be seen at concentrations as low as 0.5 μmol/L (Figure 2 to 4), a concentration found in some human plasma. This raises the possibility that EKODE is a regulator of aldosterone production in vivo. This postulate is supported by the correlation between plasma EKODE levels and plasma aldosterone in a single cohort of 24 human subjects. On the other hand, maximal stimulation by EKODE in vitro requires 10\(^{-6}\) mol/L, a concentration higher than any we have found in humans to date, casting doubt on a postulated role for EKODE as an important regulator of aldosterone in all humans. Furthermore, some circulating EKODE is bound to albumin, although the extent and strength of that binding is unknown. Despite these doubts, EKODE may affect aldosterone production in those people in whom it circulates at high concentrations, or in whom it is formed in the adrenal gland itself.

Lipoxygenase activity in the adrenal cortex has been shown to be important for the steroidogenic response to angiotensin II, but this importance is attributed to oxidized derivatives of arachidonic acid.\(^2\)\(^4\) Perhaps oxygenated derivatives of linoleic and other 18-carbon fatty acids also behave as autacoids in the adrenal.

It should be noted that other oxidized derivatives of linoleic acid produced by our cell-free reaction sequence share the adrenal properties of EKODE. Principal among these other derivatives is 13-keto-9,11-octadecadienoic acid, and even linoleic acid itself displays some activity in the adrenal gland (data not shown). The biological properties of oxidation products of all unsaturated fatty acids deserve further study. Gamma-linolenic acid (C18:3, n-6) and docosahexaenoic acid (C22:6, n-3) have been shown to inhibit aldosterone production in hypertensive rats.\(^2\)\(^5\)\(^26\) Although those inhibitory effects were originally attributed to the unmodified fatty acid, it is possible that oxidized products of the fatty acids were also active.

Oxidative reactions involving polyunsaturated fatty acids are part of a phenomenon called oxidative stress, in which reactive oxygen species accumulate and alter molecules outside of their normal metabolic sequence. Reactive oxygen species have a very short lifespan, measured in seconds or fractions of a second. Oxidative stress has been implicated in the pathogenesis of hypertension.\(^8\)\(^9\)\(^27\)\(^–\)\(^30\) Hypertension is a condition that lasts for years, so it is difficult to see how evanescent atoms or molecules could cause it. Oxidized derivatives of fatty acids might be among the long-lived products of oxidative stress that would serve as downstream mediators affecting blood pressure. After intravenous infusion into Rhesus monkeys, EKODE decayed from the plasma at a rate measured in minutes (Goodfriend, unpublished observations, 2003). That would make EKODE a more durable player in blood pressure regulation than the oxidants themselves. If EKODE proves to be an effective stimulus of aldosterone secretion in humans, then the longevity of that steroid would further extend the duration of reactive oxygen’s effect on blood pressure. Considering aldosterone’s growing list of extrarenal effects, EKODE might participate through aldosterone in the pathogenesis of other cardiovascular diseases.\(^3\)\(^1\) Also plausible is the possibility that EKODE is a regulator of aldosterone, not a mediator of its effects.

We saw positive correlations between obesity and EKODE, EKODE and aldosterone, and aldosterone and blood pressure in blacks. That would support our original hypothesis about the possible link forged by EKODE to connect obesity and hypertension. However, all of those connections were not found in the white subjects. In the same cohort, the black subjects displayed more evidence of oxidative stress, measured as isoprostanes, than the white subjects when they received an intravenous infusion of lipid.\(^1\)\(^7\)

**Perspectives**

Our findings suggest that oxidation products of polyunsaturated fatty acids other than arachidonic acid exert effects on humans that may contribute to regulation of blood pressure by their effects on aldosterone secretion. The effects may be greater in blacks than whites. At the least, these fatty acid derivatives, readily demonstrated in human plasma, merit study as possible mediators or markers of oxidative stress and its sequelae.

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